Ethanol Tolerance of *Saccharomyces cerevisiae*, L1400 (Exp #1)

John Carpenter

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Purpose:

To determine if the inoculum propagation conditions have any subsequent effects on the ability of the yeast to tolerate ethanol.

Background:

Several literature sources have considered the growth conditions of the inoculum and the subsequent effect these conditions have on the ability of the organism to ferment in the presence of ethanol. Two important factors in the SSF process thought to effect the fermentative ability of the yeast are the temperature at which the inoculum is grown and the acclimation of the inoculum to higher ethanol concentrations prior to fermentation. Higher process temperatures have been shown to increase ethanol toxicity resulting in loss of viability, depression of growth, and therefore poor fermentative performance. Likewise, an increase in ethanol concentration can shift the activation temperature for thermal death and the maximum and optimum temperatures for growth downward.' Adaptation of the inoculum to higher temperatures and ethanol concentrations may increase the initial viability and reduce the lag phase in SSF fermentations in which exponential death occurs before growth begins.

Experimental Design:

The ability of the yeast L1400 to convert glucose to ethanol and the productivity rates associated with that conversion in the presence of exogenous ethanol need to be established. This information will become important in the process design of the scaled up fermentation , recycling scheme, and downstream processing It was decided to approach the problem with a three part experimental design to address the following questions:

Experiment 1: Determine the optimal conditions for temperature and ethanol adaptation for inoculum preparation. This would be done by comparing the established growth temperatures of inoculurn at 30° C and 34° C. The second variable was adaptation to higher ethanol by fermenting to an increased ethanol concentration prior to inoculation. A reasonable level of ethanol to be produced in an inoculum is 2% (w/w) and with additional glucose and fermentation time 4% (w/w) could be expected and would bring the level close to that in the final fermentation stage.

Experiment 2: Determine the exogenous ethanol tolerance of the organism. Combining the results of experiment one, experimental data reported by Amoco [2], and literature articles, an experiment was designed to test the ability of the Labatt 1400 yeast to ferment glucose in the presence of increased ethanol concentrations by monitoring glucose consumption and ethanol production rates. The test levels chosen were 0, 5, 6, 8, and 10 % (w/w) ethanol.

Experiment 3: Investigate means of increasing the ethanol tolerance of the organism by nutrient supplementation and temperature. The literature in this area of research has investigated many additives which reportedly increased the ethanol tolerance of yeast and bacteria. Three additives chosen were phosphatidylcholine-albumin complex, soy flour, and YEP media at twice the standard concentration. Two additional experiments addressing temperature during fermentation rather than nutrient supplementation were included with the low limit at 30" C and the high limit at 34° C.

This report discusses the results from experiment 1.

Materials and Methods:

The organism was *Saccharomyces cerevisiae,* Labatt 1400 strain and is a spheroplast fusion product of the polyploid brewing strain *Saccharomyces uvarum* , strain 21 and a

genetically constructed diploid *Saccharomyces diastaticus*, strain 1384.[3] The organism was supplied by *Amoco* Corporation.

The first stage inoculum was grown from a frozen stock vial in a 250 ml baffled shake flask for 16 hours in a rotary shaker at 30° C and 150 r.p.m. The media consisted of 100 ml of 1% (w/w) CSL and 1% (w/w) glucose. This inoculum was used to inoculate a second stage consisting of two 500 ml baffled shake **flasks.** The two flasks contained 90 ml of media composed of 2% (w/w) CSL and **5%** (w/w) glucose. These two **flasks** fermented for 12 hours, one at 30° *C* and the other at 34° C, and then used to inoculate one additional flask for each temperature. Additional glucose (50 g/L) was added to the two older inoculum flasks and all four were fermented for **24** hours. The four conditions for the second stage inoculurn growth are summarized in the Table 1 below:

TABLE 1. *Conditions for the second stage inoculum.*

Conditions	Target Ethanol (g/l)	Temperature (° C)	Growth Time (hr.)	Total Glucose added (g/l)
High EtOH 30" C	40	30	36	100
High EtOH 34° C	40	**34**	36	100
LowEtOH 30" C	20	30	**24**	*50*
LowEtOH 34° C	20	**34**	24	**50**

Each of the inoculum grown under the four conditions were used to inoculate three flasks for the main fermentation (see below). Cell concentrations were determined on all four flasks using a hemacytometer. The appropriate volume of inoculum was used to give an initial cell concentration of 4×10^6 cells/ml. This number of cells corresponded to the number of cells present in 10 ml of the second stage inoculum grown at low ethanol and 30° C which had the lowest cell concentration. The difference between the inoculum volume needed to attain the target cell concentration and 10 ml was made up by adding the appropriate amount of sterile water. Each inoculum was analyzed to determine colony forming units to ensure uniformity.

The media used in the main fermentation contained 90 ml of 2% (w/w) CSL, 5% (v/v) ethanol, and 5% (w/w) glucose and 10 ml inoculum to bring the total working volume to 100 ml. The SSFs were carried out at 30° C and 34° C, corresponding to the inoculum temperature, in *250* ml baffled shake flasks capped with anaerobic gas locks. The speed of the rotary shaker was 150 r.p.m.. Seven samples were taken during the fermentation and analyzed for glucose and ethanol on a HPLC and gas chromatograph respectively.

Results and Discussion:

It was discovered that the seed inoculum used in this study had poor growth performance which compromised the first part of this experiment and resulted in excessive lag during the inoculum preparation. This led to much lower than expected ethanol concentrations being produced in the inoculum for the allotted growth time. The expected 20 g/l level attained 0.75 g/l and the 40 g/l level attained only 1.5 g/l and will be referred to as the low or high level of ethanol respectively from here on out. This lack of ethanol narrowed the range significantly and it is doubtful any differences can be detected due to the effect of ethanol.

The glucose consumption rates of all four conditions tested in the main fermentations were very similar during the first 10 hours of fermentation (see graph 1). During the fermentation period from 10 to 24 hours, the inoculum condition of low ethanol, 34° C showed a slightly higher glucose uptake rate over the other fermentation conditions. Given the small difference in ethanol concentrations it might be expected that the high ethanol, 34" C would also show an increase but the longer growing time of the inoculum may have contributed to a depressed rate. The inoculurn conditions of high ethanol, 34° C and low ethanol, 30" C were similar to each other and high ethanol, 30° C had the slowest uptake rate. The uptake rates converged over the next 24 hours and showed no difference after 48 hours fermentation time. These small variations in the uptake rate do not seem to be a significant indicator of fermentation performance.

All four conditions showed a lag in ethanol production during the first 8 hours of fermentation. After eight hours the rate became positive for all of the conditions tested (see graph 2). The productivity rates for ethanol exhibited similar patterns as the uptake rates of glucose for the four inoculum conditions tested.

The inoculum conditions with the lower ethanol concentrations (0.75 g/L) seemed to have slightly better productivity rates than the higher ethanol concentrations (1.5 g/L). The difference in the ethanol concentration was so slight leading me to believe that the difference in productivity rates was due to some other factor such as the growth phase of the inoculurn. These differences may be more pronounced if wider ranges were used for the conditions tested. There was no significant productivity for any of the conditions tested after 48 hours. The fermentation periods with increasing productivity rates are listed for the four inoculum conditions in Table 2 below:

TABLE 2. *Ethanol Productivity's (g/L h).*

Time Period (hours)	Low EtOH, 34° C	High EtOH, 30° C	High EtOH, 34° C	Low EtOH, 30° C
10 *to* 25	0.74	0.19	0.37	0.43
25 to 48.5	0.31	0.70	0.66	0.54

Conclusions:

The data in this experiment demonstrates that the temperature at which the inoculum is grown does not affect the subsequent ability of the organism to ferment glucose to ethanol. The difference in ethanol concentration was very small and it is doubtful that this level of ethanol had any subsequent effect. The level of confidence could be increased by repeating this experiment with wider ethanol ranges and ensuring that inoculum in the same growth phase is used in all tests.

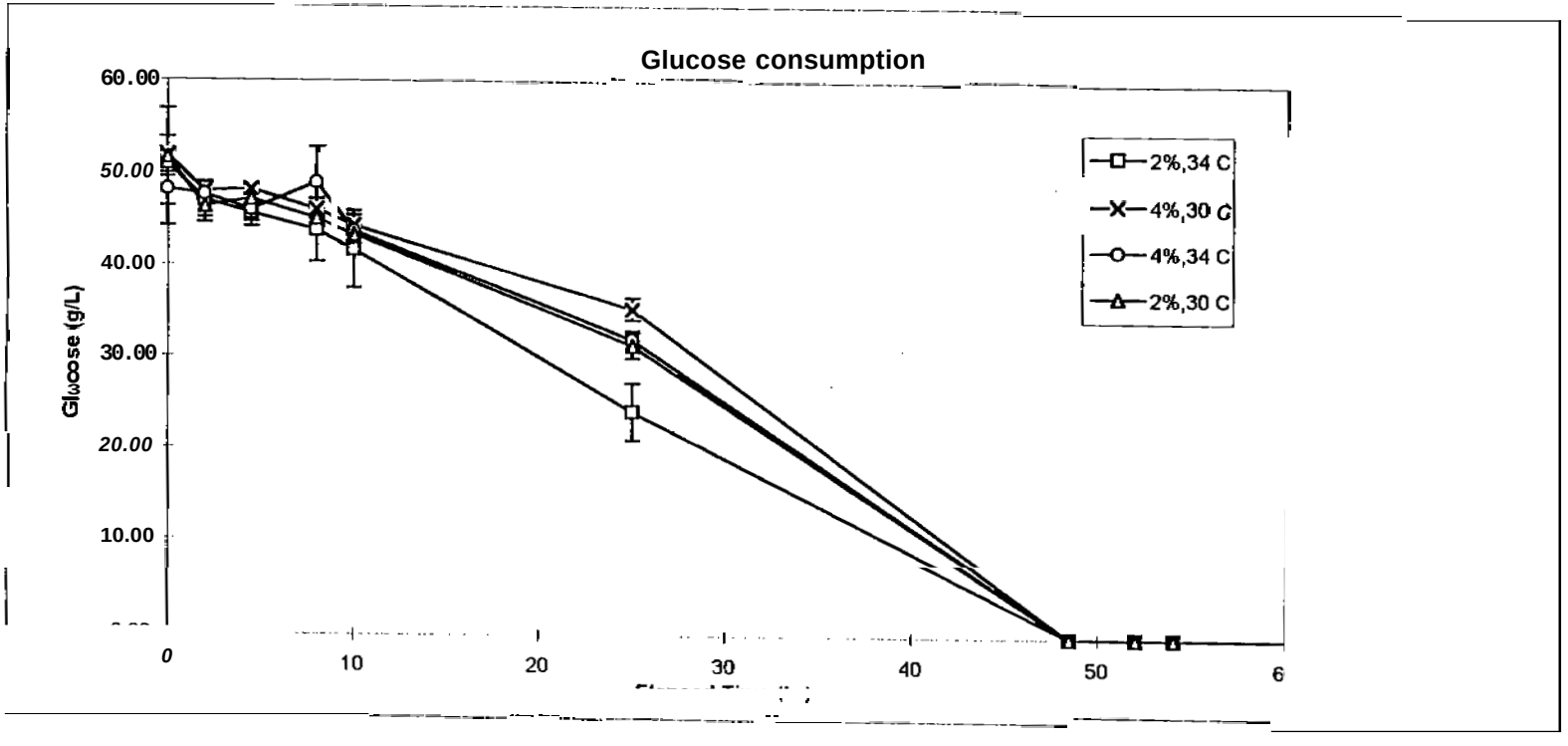
Bibliography

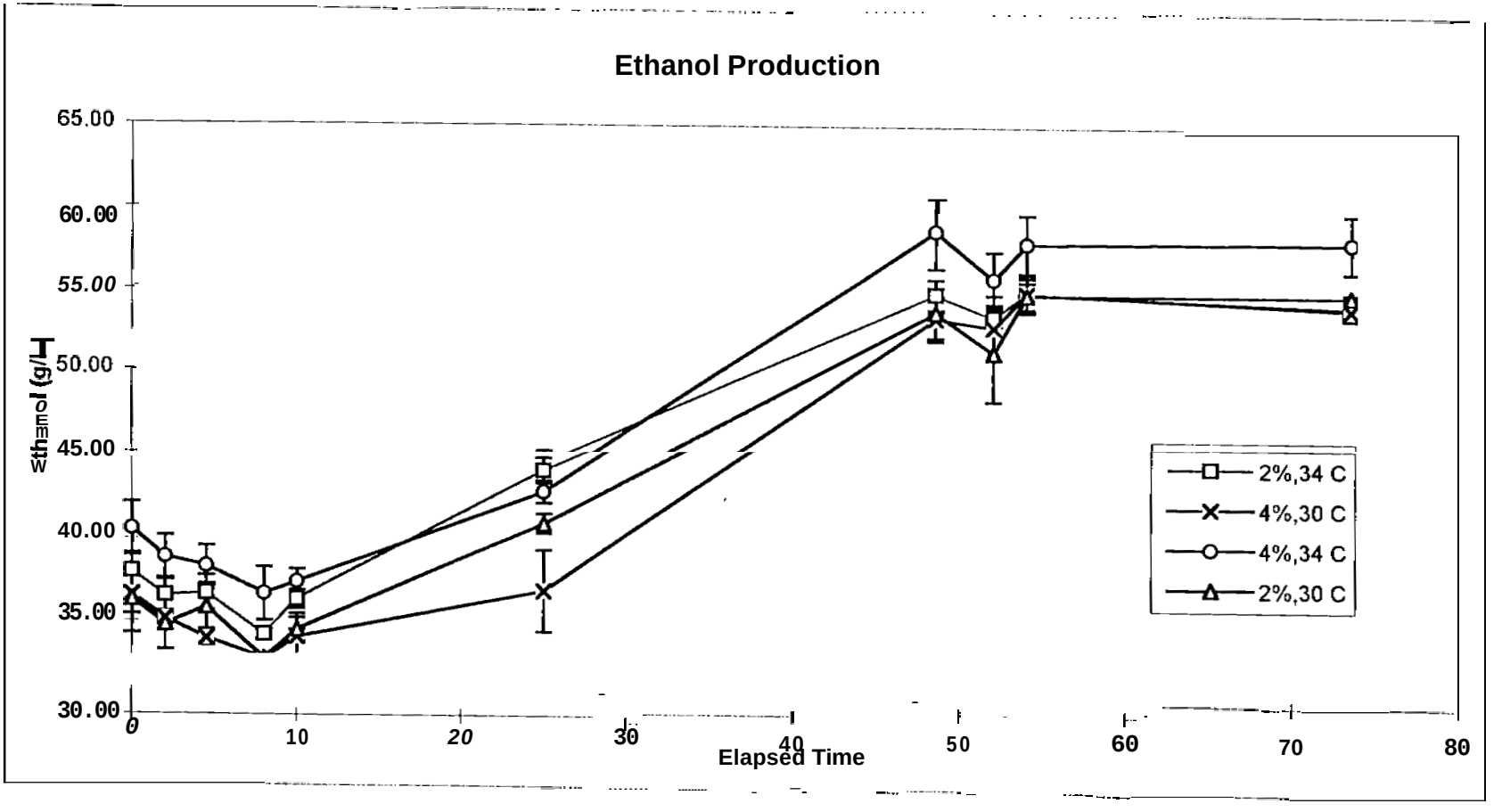
1. Van **Uden,** N. ***Annual Report of Fermentation Processes 8,*** **11(1985).**

2. Mitchell, L. Labatt **1400** Ethanol Tolerance: Amoco run **report 505-038.** (1**994).**

3. Panchal, C. **J.,** Harbison, **A., Russell, I. & Stewart,** ***G. G. Biotechnology Letters*** **4,** ***33-38*** (1**982).**

Glucose


Glucose consumption
60.00
50.00
40.00
30.00
20.00
10.00
Glucose (g/L)
0
10
20
30
40
50
2%,34 C
4%,30 C
4%,34 C
2%,30 C


Ethanol Production
65.00
60.00
55.00
50.00
45.00
40.00
35.00
30.00
Ethanol (g/L)
0
10
20
30
40
50
60
70
80
Elapsed Time
2%,34 C
4%,30 C
4%,34 C
2%,30 C

Graph 2

Glucose Data

			2%,34 C			
Time sample	Elapsed Time	Flask 7	Flask 9	Flask 11	AVG	STDDEV
0	0	50.9	~~48.4~~	51	50.95	1.47
1	2	45.7	~~44.6~~	48.3	47.00	1.90
2	4.5	46	~~44.2~~	45.2	45.60	0.90
3	8	43.5	~~37.9~~	44.1	43.80	3.42
4	10	41.6	~~34.3~~	41.7	41.65	4.24
5	25	21.8	~~<0.1~~	26.2	24.00	3.11
6	48.5	<0.1	~~<0.1~~	<0.1	0.00	0.00
7	52	<0.1	~~<0.1~~	<0.1	0.00	0.00
8	54	<0.1	~~<0.1~~	<0.1	0.00	0.00
9	73.5	<0.1	~~<0.1~~	<0.1	0.00	0.00
			4%,30 C			
Time sample	Elapsed Time	Flask 2	Flask 3	Flask 5	AVG	STDDEV
0	0	50.3	51.2	54	51.83	1.93
1	2	48.9	47.6	47.4	47.97	0.81
2	4.5	48.3	48.4	47.7	48.13	0.38
3	8	24.9	45.3	46.8	46.05	1.06
4	10	43.2	45.4	44.2	44.27	1.10
5	25	36.3	35.3	33.9	35.17	1.21
6	48.5	<0.1	<0.1	<0.1	0.00	0.00
7	52	<0.1	<0.1	<0.1	0.00	0.00
8	54	<0.1	<0.1	<0.1	0.00	0.00
9	73.5	<0.1	<0.1	<0.1	0.00	0.00
			4%,34 C			
Time sample	Elapsed Time	Flask 8	Flask 10	Flask 12	AVG	STDDEV
0	0	43.6	50.5	50.4	48.17	3.96
1	2	46.8	48.8	47.3	47.63	1.04
2	4.5	43.9	47.2	46.7	45.93	1.78
3	8	47.6	53.3	45.9	48.93	3.88
4	10	43.5	44.3	43.5	43.77	0.46
5	25	32.2	30.8	32.6	31.87	0.95
6	48.5	<0.1	<0.1	<0.1	0.00	0.00
7	52	<0.1	<0.1	<0.1	0.00	0.00
8	54	<0.1	<0.1	<0.1	0.00	0.00
9	73.5	<0.1	<0.1	<0.1	0.00	0.00
			2%,30 C			
Time sample	Elapsed Time	Flask 1	Flask 4	Flask 6	AVG	STDDEV
0	0	45.8	53	56.1	51.63	5.28
1	2	48.2	45.2	45.4	46.27	1.68
2	4.5	45.2	48.1	48.1	47.13	1.67
3	8	44.1	46	45.1	45.07	0.95
4	10	42.2	44.1	43.6	43.30	0.98
5	25	29.8	32.6	31.2	31.20	1.40
6	48.5	<0.1	<0.1	<0.1	0.00	0.00
7	52	<0.1	<0.1	<0.1	0.00	0.00
8	54	<0.1	<0.1	<0.1	0.00	0.00
9	73.5	<0.1	<0.1	<0.1	0.00	0.00

Ethanol Data

		2%,34 C				
Time sample	Elapsed Time	Flask 7	Flask 9	Flask 11	AVG	STDDEV
0	0	38.3	36.8	38.9	38.00	1.08
1	2	36.2	35.8	37.6	36.53	0.95
2	4.5	36	36.2	37.9	36.70	1.04
3	8	34.3	33.9	34.5	34.23	0.31
4	10	36.6	36.7	35.7	36.33	0.55
5	25	44.3	45.1	43.5	44.30	0.80
6	48.5	54	54.5	55.8	54.77	0.93
7	52	53.9	52.8	53.5	53.40	0.56
8	54	53.7	55.2	55.6	54.83	1.00
9	73.5	54.6	53.3	54.4	54.10	0.70
		4%,30 C				
Time sample	Elapsed Time	Flask 2	Flask 3	Flask 5	AVG	STDDEV
0	0	36.6	35.4	37.7	36.57	1.15
1	2	35.4	35	35.1	35.17	0.21
2	4.5	34.3	34.2	33.5	34.00	0.44
3	8	33	32.6	32.8	32.80	0.20
4	10	33.2	35.7	33.4	34.10	1.39
5	25	38.1	38.6	34.1	36.93	2.47
6	48.5	53.6	51.8	54.5	53.30	1.37
7	52	50.5	54.2	53.6	52.77	1.99
8	54	54.4	54	56.2	54.87	1.17
9	73.5	53.7	54.1	54.1	53.97	0.23
		4%,34 C				
Time sample	Elapsed Time	Flask 8	Flask 10	Flask 12	AVG	STDDEV
0	0	41	38.8	42	40.60	1.64
1	2	37.4	39.8	39.5	38.90	1.31
2	4.5	37	38.5	39.5	38.33	1.26
3	8	35.1	36.6	38.3	36.67	1.60
4	10	37	36.9	38.3	37.40	0.78
5	25	43.4	42.2	43.4	43.00	0.69
6	48.5	57.5	57.1	61.2	58.60	2.26
7	52	55.2	54.4	57.6	55.73	1.67
8	54	56.2	57.7	59.7	57.87	1.76
9	73.5	57.1	56.9	60	58.00	1.73
		2%,30 C				
Time **sample**	Elapsed Time	Flask 1	Flask 4	Flask 6	AVG	STDDEV
0	0	34.1	36.7	**38.1**	36.30	**2.03**
1	2	35.31	**36.1**	**33.1**	**34.83**	**1.55**
2	4.5	37.41	35.4	34.8	35.87	**1.36**
3	**8**	32.7	**33.1**	32.6	32.80	**0.26**
4	10	33.8	35.21	**34.5**	34.53	0.70
5	25	40.5	40.91	41.7	**41.03**	0.61
6	48.5	54.3	51.9	54.8	53.67	1.55
7	52	47.8	**53.1**	52.8	51.23	2.98
8	54	55.5	54.9	53.9	54.77	0.81
9	73.51	54.9	54.9	54.6	54.80	**0.17**